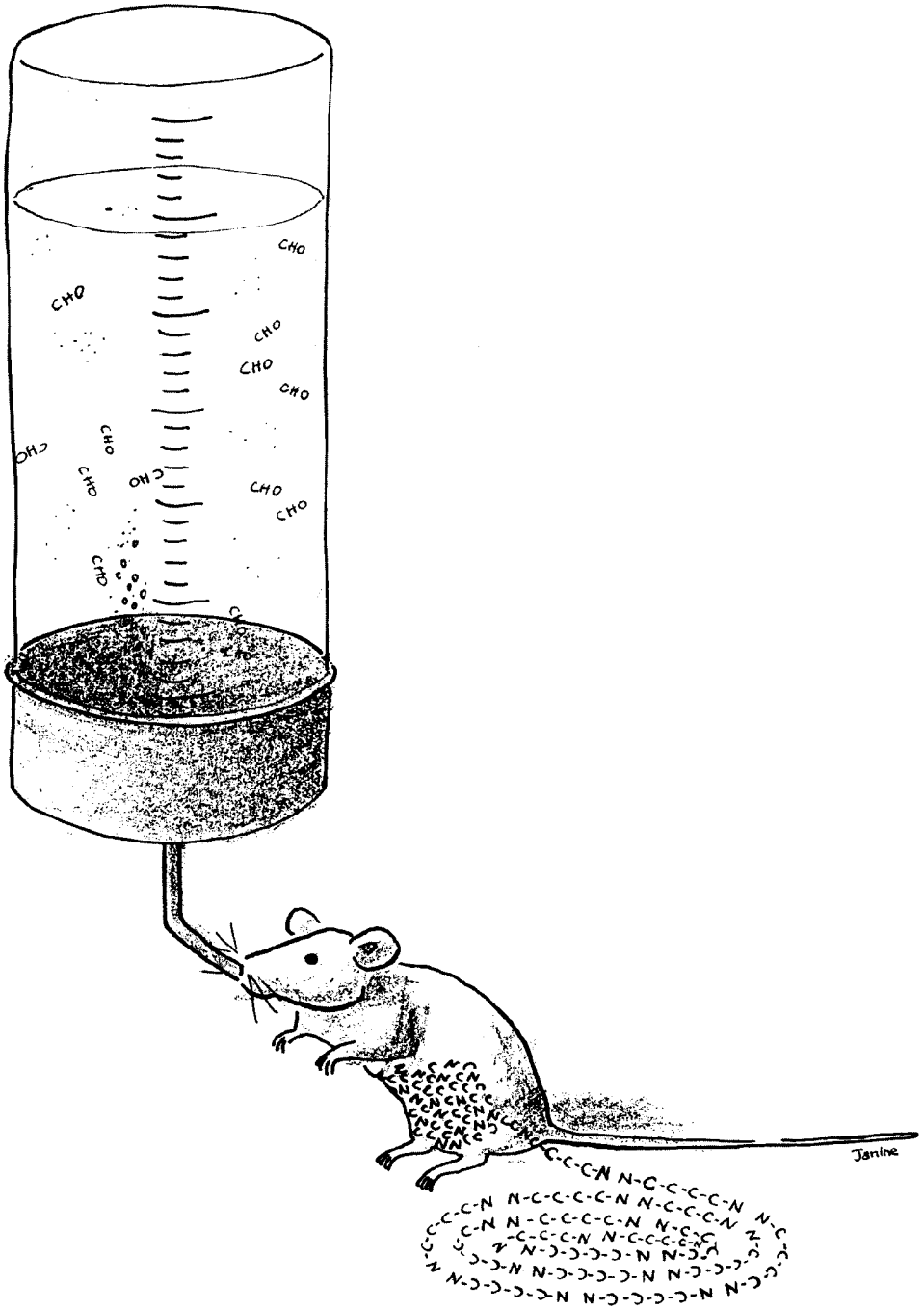
CHAPTER 2

POLYAMINES FROM THE GASTROINTESTINAL TRACT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2.1 Microbial influences on urinary polyamine excretion

H. P. W. M. Satink, J. Hessels, A. W. Kingma, G. A. van den Berg, F. A. J. Muskiet and M. R. Halie.

Summary

We determined diamines, polyamines, their monoacetylated conjugates and some of their catabolites in urines of healthy persons during decontamination of the gastrointestinal tract and patients with urinary tract infections. The compounds were also measured after in vitro incubation of urines from healthy persons and patients. During decontamination the urinary excretion of total putrescine decreased by a small amount. This fall was for the greater part accountable to monoacetylated putrescine. Free putrescine levels were increased in urines of patients with urinary tract infections, decreased after therapy and increased after incubation of the pretherapeutical samples. Total cadaverine decreased during decontamination and increased during recontamination. The changes were partly accountable to monoacetylated cadaverine. Free cadaverine levels of patients with urinary tract infections were normal and did not change after therapy. These data show that, under normal conditions, a small part of monoacetylated putrescine and a considerable part of monoacetylated cadaverine originate from the gastrointestinal tract, and that urinary tract infections lead to an increase of free putrescine. The microbial synthesis of putrescine in the gastrointestinal- and urinary tracts, should therefore be taken into account for the interpretation of urinary putrescine levels as a parameter for body cell turnover.

Introduction

The diamine putrescine (Pu) and polyamines spermidine (Sd) and spermine (Sp) are low molecular mass cations that are ubiquitous in nature. Most probably by their association with nucleic acids they are indissolubly united with growth processes. In this connection the relation between intra- and extracellular polyamine concentrations and (tumor) cell turnover has been studied intensively.[1]

Much less is known about the diamine cadaverine (Cad), which is likewise a constituent of living cells. However, there is evidence that Cad present in human body fluids may be of prokaryotic origin.[2-4] Concentrations of Cad in hydrolysed urines decreased by about 70 % during selective decontamination of the gastrointestinal tract[4], suggesting that it is mainly synthesised by aerobic bacteria. In the urine of healthy persons Cad is excreted both in the free and monoacetylated (acCad) form.[5] In a previous study we suggested that the excretion profiles of free Cad and acCad encountered during chemotherapy of patients with high grade non-Hodgkin's lymphoma are related to variations in the composition and extent of the microbial flora in the gastrointestinal and urinary tracts.[5]

Cad may also be formed in mammalian cells.[6-8] It is the decarboxylation product of lysine, catalysed by lysine decarboxylase (LDC; EC 4.1.1.18). However, it has never been demonstrated that mammalian cells contain LDC activity. Its synthesis may take place during periods of high ornithine decarboxylase (ODC; EC 4.1.1.17) activity.[9] However, ODC is a highly specific enzyme that catalyses the formation of Pu from ornithine in a strictly regulated manner. Under normal conditions it has not yet been shown that lysine is an appropriate substrate.[6]

Polyamines and their monoacetylated conjugates are degraded by a large number of enzymes.[10] The quantitatively most important catabolic pathway for the free diamines is their oxidative deamination by copper-containing amine oxidases, yielding amino aldehydes. The latter are subsequently oxidised to non-α-amino acids.[11,12] Intraperitoneal injection of deuterated free Cad in rats led to the appearance of both labelled total Cad and δ-amino valeric acid (δ-Val) in urine.[11] In addition

δ-Val was identified in the urine of normal persons and patients with malignancies.[12]

In an effort to define more clearly the origin of urinary diamines, we investigated the urinary diamine and polyamine excretion by healthy persons during selective and subsequent total decontamination of the gastrointestinal tract and by patients with urinary tract infections before and after therapy. We also analysed *in vitro* incubated urines of the patients with urinary tract infections prior to therapy and apparently healthy controls.

Materials and methods

Urine samples

Early morning urine samples from adult patients with urinary tract infections before (31 specimens) and after successful therapy (21 specimens) were collected by two general practitioners. Portions were kept at 4 °C and used for incubation experiments (see below) within 2 h after collection. A portion of each urine sample was centrifuged for 10 min at 1700 x g and 4 °C. The supernatant was stored at -20 °C until analysis.

Urine samples from 12 apparently healthy persons (early morning specimens) and 8 participants in the decontamination experiment (24 h specimens) were collected. During collection of the 24 h urines the samples were kept refrigerated. Portions of the normal early morning urine specimens were used for incubation experiments (see below). The remainders and portions of 24 h urines were stored at -20 °C until analysis.

Experiments

In vitro incubation of urine samples

Within 2 h after voidance urine portions from 18 patients with urinary tract infections and 12 from healthy adult controls were used for subsequent incubation during 20 h at 37 °C to stimulate microbial growth. After incubation the samples were centrifuged at 1700 x g for 10 min. Their supernatants were stored at -20 °C until analysis.

Patients with urinary tract infections

Urinary tract infections were diagnosed on the basis of the symptoms usually experienced during such conditions, microscopic inspection of the urinary sediment and by a semi quantitative assay of urinary nitrite, using a stick that operates via the principle of azo-dye formation (Nitur test; Boehringer Mannheim; Almere, The Netherlands).[13] None of the urines of the healthy controls showed a positive nitrite test.

Gastrointestinal decontamination of healthy persons

Eight apparently healthy persons participated in an experiment comprising decontamination of the gastrointestinal tract for 8 consecutive days. From day 3 to 11 they underwent selective (aerobic) decontamination by oral intake of polymyxin B (4 x 200 mg/day).[4,14] From day 7 to 11 cephaloridine (4 x 500 mg/day, oral) was also taken to reduce the number of anaerobic bacteria.[4] To prevent the overgrowth of yeasts amphotericin B (4 x 500 mg/day, oral) was taken during total decontamination. After treatment the volunteers were allowed to recontaminate. This protocol was approved by the Medical Ethical Committee of the State University of Groningen (MEC 83/3/44).

For a check on the effect of the decontamination regiments faecal portions were collected at days 2, 6, 10 and 14 and cultured using standard bacteriological procedures. The number of aerobic bacteria per gram of faeces amounted to about 10^4, 1, 10 and 10^6 and those of anaerobic bacteria to 10^{11}, 10^{11}, 10^5 and 10^5, respectively. A detailed report on the results of the quantitative and qualitative studies of the faecal aerobic and anaerobic bacteria, as derived from the present experiment, has been published by Keyzer et al.[4]

Analytical methods

Determination of free polyamines, their monoacetylated conjugates and some free catabolites

The free (nonconjugated) polyamines diaminopropane (DAP), Pu, Cad, Sd and Sp, their monoacetylated conjugates N-acetylputrescine (acPu), acCad, N^1-acetylspermidine (N^1-acSd), N^8-acetylspermidine (N^8-acSd) and N^1-acetylspermine (N^1-acSp), and the free catabolites putreanine (Putr), isoputreanine (Isoputr) and γ-lactam form of isoputreanine (Isoga) were isolated from 1 ml of urine by adsorption onto silica. After conversion into their N-heptafluorobutyryl methyl esters they were determined by means of capillary gas chromatography with nitrogen-phosphorus detection, as previously described.[5]

Determination of total polyamines and their non-α-amino acid catabolites

The total polyamines concentrations of DAP, Pu, Sd, Isoputr, and Sp were determined in acid-hydrolysed urines of the participants in the decontamination experiment by a similar method.[11] From the passage of the silica column, δ-Val was isolated by means of cation exchange chromatography and converted into its N-heptafluorobutyryl isobutyl ester.[15] Quantification was performed by gas chromatography with nitrogen-phosphorus detection, as previously described.[15]

Statistics

The concentrations of polyamines, monoacetylated polyamines and their catabolites were expressed in relation to those of creatinine.[16] Data sets were compared using unpaired and paired Student's t tests. In case of non-Gaussian distribution of one or both of the compared data sets the Wilcoxon-test was employed (marked *). P values ≤ 0.05 (one-sided) and ≤ 0.025 (two-sided) were considered significant. Differences between data sets obtained on different occasions were expressed as a proportional increase or decrease of their mean concentrations.

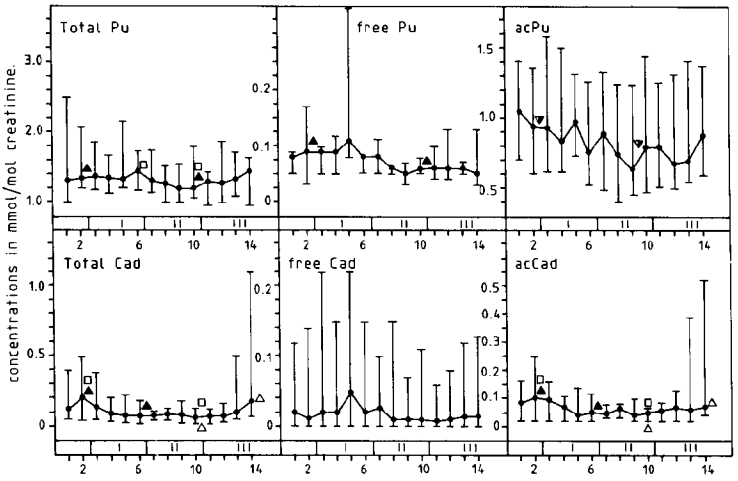

Figure 1. *Median urinary concentrations and ranges for total Pu, free Pu, acPu, total Cad, free Cad and acCad.*

Diamines were measured during selective- (I), and total (II) decontamination and during spontaneous recontamination (III) of the gastrointestinal tract. Concentrations are expressed in mmol/mol creatinine. Matching signs indicate significant differences.

Results

Gastrointestinal tract decontamination of healthy persons

Figure 1 depicts median urinary concentrations and ranges of total Pu, free Pu, acPu, total Cad, free Cad and acCad before, during and after decontamination of the gastrointestinal tract. Days 2, 6, 10 and 14 were compared.

Total Pu levels decreased (about 12%) during total decontamination (days 2-10: $p = 0.02$ *; days 6-10: $p = 0.017$ *). Free Pu concentrations decreased ($p = 0.02$) from days 2-10, while acPu levels dropped ($p = 0.004$) when days 2-9 were compared. The major part (about 90%) of the fall in total Pu was accountable to acPu.

Total Cad decreased (about 65%) between days 2-6 ($p = 0.025$ *), and days 2-10 (about 72%; $p = 0.01$). This decrease was for about 35% accountable to a fall in acCad (days 2-6: $p = 0.068$; days 2-10: $p = 0.02$). The concentrations of δ-Val (median 0.24 mmol/mol creatinine;

TABLE I: Median polyamine concentrations and range (mmol/mol creatinine) of patients with urinary tract infections before and after successful therapy and after in vitro incubation of urine samples collected before therapy.

Analyte	Before therapy (n=31)		After therapy (n=21)		After in vitro incubation[g]	
	Median	Range	Median	Range	Median	Range
Free DAP	0.10[a]	n.d.-0.58	0.09[a]	n.d.-0.32	0.04	n.d.-0.51
Free Pu	0.65[bc]	0.06-3.30	0.13[b]	n.d.-3.41	2.27[c]	0.05-7.57
acPu	1.09[b]	0.35-2.96	0.85	n.d.-2.33	1.35[d]	0.48-3.37
Free Cad	0.33[d]	0.07-4.15	0.24	n.d.-0.56	0.65[e]	0.20-6.58
acCad	0.08	n.d.-1.13	0.03	n.d.-1.41	0.18	n.d.-1.14
Isoputr[f]	0.18	n.d.-0.80	0.12	n.d.-0.44	0.14	n.d.-0.74
Putr	0.32	0.10-0.80	0.39	n.d.-2.09	0.24	n.d.-0.59
Free Sd	0.03	n.d.-0.22	0.03	n.d.-0.12	0.04	n.d.-0.22
N^1-acSd	0.36	0.03-0.91	0.34	0.08-1.08	0.37	0.11-0.81
N^8-acSd	0.26	0.03-0.73	0.26	n.d.-0.54	0.28	0.07-0.50
Free Sp	0.04	n.d.-0.88	0.02	n.d.-0.12	0.03	n.d.-0.52

[f]Isoputr = free Isoputr + Isoga. [g]Pretherapeutic urine samples (n = 18) were incubated for 20 h at 37 °C, within 2 h after voidance. AcDAP and N^1-acSp were occasionally detectable; n.d., not detectable; [a]p = 0.047*; [b]p = 0.006*; [c]p = 0.039; [d]p = 0.017*; [e]p = 0.005*. For other abbreviations see text.

range: 0.04-1.52 mmol/mol creatinine) fluctuated during the whole observation period, but did not show any correlation with total-, free- or acCad levels. During recontamination (days 10-14) increases were observed for total Cad (about 370%; p = 0.012 *) and acCad (about 250%; p = 0.025). The increase of total Cad could for about 47% be ascribed to acCad.

No changes were found in the concentrations of the other polyamines, their conjugates and metabolites.

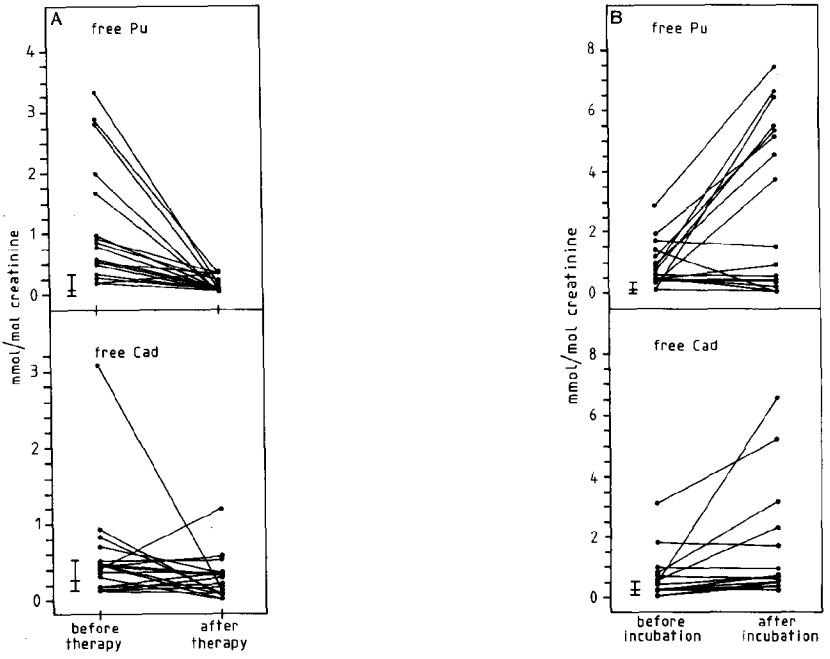

Figure 2. *Urinary free Pu and free Cad concentrations before and after successful treatment of patients with urinary tract infections (A), and before and after in vitro incubation of pretreatment samples (B).*

Samples were incubated for 20 h at 37 °C. The bars indicate the normal median values and ranges as established in the present study. Concentrations are expressed in mmol/mol creatinine.

Patients with urinary tract infections

Table I lists the complete set of median polyamine data and ranges for urine samples from patients with urinary tract infections before and after successful treatment and after in vitro incubation of pretherapeutically collected samples. Figure 2 visualises the patient-individual effects of successful treatment of urinary tract infections (Fig. 2A) and *in vitro* incubation of pretreatment samples (Fig. 2B) on the concentrations of free Pu and free Cad.

When compared with urinary polyamine concentrations of the control group (Table I vs. Table II) untreated patients with urinary tract infections had increased free DAP ($p = 0.012$), free Pu ($p = 0.003$), acPu ($p =$

TABLE II: Median urinary polyamine concentrations and range (mmol/mol creatinine) of twelve healthy adult controls before and after *in vitro* incubation, in relation to the previously established normal range.

Analyte	Before *in vitro* incubation		After *in vitro* incubation		Normal values (see ref.[5])	
	Median	Range	Median	Range	Mean	Range
Fee DAP	n.d.*	n.d.-n.d.	0.06*	n.d.-0.09	0.16	0.02-0.69
Free Pu	0.05	n.d.-0.37	0.08	0.02-2.13	0.35	0.08-1.06
acPu	0.72[b]	0.42-1.08	0.79[b]	0.56-1.44	1.18	0.58-2.25
Free Cad	0.23	0.12-0.49	0.28	0.12-0.53	0.40	0.01-0.82
acCad	0.10	n.d.-0.28	0.09	n.d.-0.25	0.16	0.04-0.87
Isoputr[f]	0.13	0.07-0.16	0.14	0.11-0.18	0.64	0.18-1.20
Putr	0.20[c]	0.09-0.26	0.27[c]	0.17-0.32	0.24	0.12-0.36
Free Sd	0.03	0.01-0.04	0.04	0.02-0.05	0.05	0.01-0.09
N^1-acSd	0.26	0.10-0.39	0.29	0.15-0.41	0.39	0.12-0.72
N^8-acSd	0.24	0.11-0.30	0.26	0.18-0.37	0.30	0.15-0.65
Free Sp	0.08[d]	n.d.-0.23	0.14[d]	0.02-0.33	0.08	0.01-0.28

Within 2 h after voidance, urine samples were incubated for 20 h at 37 °C. [f]Isoputr = Isoputr + Isoga; n.d.:not detectable; *p = 0.018*; [b]p = 0.012; [c]p = 0.013; [d]p = 0.013*. For other abbreviations see text.

0.024), Putr (p = 0.007) and N^1-acSd (p = 0.017). Urinary free Pu levels decreased (about 65%; p = 0.006*) after successful treatment (Table I, Fig. 2A), while free Pu (about 200%; p = 0.039*) and free Cad (about 100%; p = 0.005*) increased after *in vitro* incubation of pretherapeutically collected samples (Table I, Fig. 2B). Except for some small alterations in free DAP and acPu no other therapy- or *in vitro* incubation-induced alterations were noted for urines of patients (Table I). Only small incubation-induced changes of free DAP, acPu, Putr and free Sp occurred in urines of the control group (Table II).

Discussion

Origins of free Pu and acPu

Under normal conditions the greater part of total Pu is excreted as acPu (Table II). During decontamination of healthy individuals total-, free- and acPu decreased (Fig. 1). The vast majority of the fall in total Pu could be attributed to acPu, which suggests that a very small part of acPu originates from the gastrointestinal tract. The remaining excretion of urinary acPu after total decontamination theoretically originates from normal body polyamine turnover, the uptake of Pu from the diet, the formation of Pu by the resident flora in the oral cavity and/or gastric lumen[2] and the remaining anaerobic flora in the intestine.

Free Pu concentrations are occasionally increased during urinary tract infections (Fig. 2A, Table I vs. Table II). After therapy free Pu levels decreased dramatically (Fig. 2A, Table I), suggesting that the pretherapeutically increased free Pu concentrations are notably related to the activity of the microbial flora in the urinary tract. The latter hypothesis becomes confirmed by the increase of free Pu levels after *in vitro* incubation of the pretherapeutically collected urine samples (Fig. 2B, Table I).

Origins of urinary free Cad and acCad

From the steep decrease of total Cad during decontamination and its increase during spontaneous recontamination (Fig. 1) it may be concluded that in healthy humans urinary total Cad originates for the greater part from the microbial flora in the gastrointestinal tract. The decrease during decontamination and the increase during spontaneous recontamination were for about 35% and 47%, respectively, accountable to acCad, which suggests that urine contains other acid-hydrolysable conjugates of Cad from gastrointestinal origin. No significant changes were observed in the urinary levels of δ-Val, which apparently is the quantitatively most important catabolite of Cad.[12] The remaining excretion of acCad and the unaltered urinary concentrations of δ-Val during total decontamination theoretically originates from the uptake of Cad from the diet[17], the

formation of Cad by the resident flora in the oral cavity and/or gastric lumen[2] and the remaining anaerobic flora in the intestine.

Urinary free Cad concentrations were not increased during urinary tract infections (Fig. 2A, Table I vs. Table II), nor were there consistent changes in free Cad levels after therapy (Fig. 2A, Table I). On the other hand, free Cad concentrations increased after incubation of pretherapeutic urine samples (Fig. 2B, Table I). The latter observation suggests that an increase of free Cad may be caused by the activity of the microbial flora in the urinary tract.

Microbial decarboxylation of ornithine and lysine in the gastrointestinal and urinary tracts

Microbial amino acid decarboxylases, such as ODC and LDC, can be induced by lowering the pH of the culture medium. Microbial synthesis of free Pu and free Cad may therefore, at least partly, be a mechanism that controls the extracellular pH, most probably by the uptake of protons.[18]

The present experiments indicate that in the urine of healthy adults a small part of acPu and a considerable part of acCad originate from the gastro-intestinal tract, and that an increase of notably free Pu can arise from the microbial flora in the urinary tract. The acetylation of free Pu and free Cad after their absorption from the gastrointestinal tract may be a normal detoxification process, as free polyamines are toxic compounds.[19] As may be concluded from the *in vitro* incubation of microbially infected urine samples, microbial amino acid decarboxylases apparently give preference to ornithine over lysine as a substrate. This occurs despite the higher normal urinary concentrations of lysine than ornithine, arginine (the precursor of ornithine) and the sum of ornithine and arginine.[20] The preference for ornithine over lysine as a substrate may at least partly be caused by inhibition of LDC by free Pu.[18]

The higher production of free Pu is probably also the case in the gastrointestinal tract. The reason that this is not reflected by a steeper decrease of urinary acPu than acCad during gastrointestinal decontamination may be that, in contrast to Cad, Pu may also be used for the synthesis of the higher polyamines Sd and Sp after its absorption.

Consequences of the microbial production of polyamines for the use of urinary polyamine levels as tumor markers

The determination of urinary polyamines is of some value in clinical oncology as polyamines and their catabolites appear to be indicators of cell turnover, at least for some selected tumors.[1,21] It is worth noting that most of the studies used analytical procedures that transform all acid-hydrolysable polyamine conjugates to their free form, and hence do not differentiate between the free and conjugated forms. We occasionally encountered very high levels of total-, free- and acPu and total-, free- and acCad in urine samples of patients with high grade non-Hodgkin's lymphoma during chemotherapy.[5,21] As an increase of these levels did not always coincide with an increase of the concentrations of those polyamines which definitely reflect cell death (such as Sd, Sp and Isoputr[5,21]), the former may, at least partly, be related to variations in the composition, extent and activity of the microbial flora in the gastrointestinal and urinary tracts.

Based on the data from patients with urinary tract infections it may be concluded that when urinary Pu levels are used as a parameter for body cell turnover, one should merely consider its acetyl conjugate. However, as suggested by the gastrointestinal tract decontamination experiments with healthy adults, urinary acPu may also originate from the microbial production of free Pu in the gastrointestinal tract. The latter origin should especially be considered in case simultaneous increases of acPu and acCad occur.

Acknowledgements

We thank Dr. J.J. Keyzer for conducting the gastrointestinal decontamination experiments and Drs H. van der Riet and R.P. Kievit for collecting urine samples of patients with urinary tract infections. We are also grateful to Mr. M. Volmer, Central Laboratory for Clinical Chemistry, for his assistance in the statistical analysis.

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2.2 Bacterial lactulose fermentation in rats increases urinary polyamine excretion

J. Hessels, E. M. Schopman, G. J. de vries, H. Ferwerda,
A. W. Kingma and F. A. J. Muskiet.

Summary

Factors that lead to the production of polyamines in the lumen of the colon of the rat were investigated. Putrescine may be produced in the colon by bacterial biodegradative decarboxylases, which are induced in acidic medium. Acidification of the colon content was pursued by enhancement of fermentation following administration of lactulose by gavage. Daily dosages of 1.3, 2.7 and 4.0 g lactulose/rat caused increased fecal putrescine levels and a, dose dependent, highly increased urinary excretion of spermidine, spermine and to a lesser extent putrescine. It is concluded that in the GI tract putrescine is formed in the process of bacterial biodegradative decarboxylation of amino acids. After its absorption it becomes partially converted into spermidine and spermine. The excess is excreted into urine. Possible implications of GI polyamine production and absorption during enhanced carbohydrate fermentation in patients with portal-systemic encephalopathy, neonates and during treatment with polyamine depleting drugs are discussed.

Introduction

Polyamines are necessary components for both eucaryotic and prokaryotic cell growth. Putrescine (1,4-diaminobutane) is synthesized by decarboxylation of ornithine by ornithine decarboxylase. Spermidine and spermine are formed from putrescine by attachment of an aminopropyl

group to one or both amino groups, respectively.[1] Spermine is found at low concentrations in particular bacteria only.[2]

Recent studies demonstrated that total gastrointestinal (GI) tract decontamination markedly improves the cytostatic effect of the polyamine biosynthesis inhibitor α-D,L-difluoromethylornithine (suicide-inhibitor of ornithine decarboxylase[3]) towards various tumors in mice.[4-7] From these results we concluded that the bacteria in the large intestine are able to synthesize putrescine, which is subsequently absorbed and transported through the body.[4]

It is known for a long time that cultured bacteria are also able to produce a variety of amines for non-structural purposes, e.g. putrescine, cadaverine, histamine and tyramine.[8-10] These products are synthesized by similar decarboxylation reactions as their structural counterparts, i.e. putrescine from ornithine or indirectly (via agmatine) from arginine, cadaverine from lysine, histamine from histidine and tyramine from tyrosine. Common features of the underlying bacterial decarboxylases are their inducibility in acidic medium in the presence of substrate, requirement of pyridoxal phosphate as a cofactor (except for histidine decarboxylase) and optimal activity at Ph 4-7.[8,11,12] To distinguish these types of decarboxylases from their biosynthetic counterparts, the former are called biodegradative decarboxylases.[9] Augmented production of putrescine by bacteria is supported by the observation that the amount of biodegradative ornithine decarboxylase in E. Coli, cultured under semi anaerobic conditions, at low pH and in rich media containing excess amino acids is much higher than the biosynthetic form (i.e. 7 % and 0.023 % of total protein, respectively).[2] The splitting of amino acids into strongly basic amines and CO_2 by biodegradative decarboxylation is thought to act as a neutralizing mechanism in unfavourably acidic environment.[9,13]

Biodegradative decarboxylation *in vivo* most probably takes place during bacterial carbohydrate fermentation. This leads to the formation of short chain fatty acids (SCFA; notably acetic, propionic and butyric acids) and lactic acid. However, in practice these acids do not cause a marked drop in cecal and colonic pH.[14] Apart from the above mentioned process

this is thought to be accomplished by their rapid absorption[15-17] and the excretion of bicarbonate into the lumen.[16,17]

Several reports have described biodegradative decarboxylation by bacteria in the GI tract.[18-21] However, it was generally believed that the produced amines are almost completely degraded in the gut, notably accomplished by oxidative deamination.[22,23] Putrescine and cadaverine can also be degraded by bacterial diamine oxidases in, e.g. Bacteroides, Bifidobacteria and Clostridia.[10] Recently, Osborne and Seidel[24] have demonstrated that putrescine and cadaverine synthesized by the colonic microflora are absorbed and transported to the proximal gut via enterohepatic circulation.

The aim of the present study was to investigate factors that lead to the production of polyamines in the lumen of the GI tract of the rat, their metabolism and their excretion into the urine after absorption. Stimulation of bacterial fermentation was achieved by oral administration of lactulose (1-O- β-D-galactopyranosyl-D-fructose), a disaccharide that is indigestible in the small intestine.[25]

Materials and methods

Animals and diets

Female Wistar rats, weighing 150-180 g, were used in all experiments. Animals were housed under standard laboratory conditions (20-22 °C, 50-60 % relative humidity, 12 h-light/12 h-dark cycle). For collection of urine and feces rats were individually housed in metabolic cages. Standard rat food (RMH-B; Hope Farms, Woerden, The Netherlands) and drinking water were provided ad libitum.

Sample collection

Twenty-four hour urines were collected in 50 ml beakers containing 1 ml 2 M HCl. Samples were stored at 4 °C until analyses (within 2 weeks). Fresh feces and cecum and colon contents from rats were collected and stored at - 20 °C until analysis for SCFA and polyamine contents (within 2 weeks).

Animal Experiments

Influence of oral lactulose administration on urinary polyamine excretion

Polyamines in urine were measured after administration of various doses of lactulose. After an adaptation period of 2 days 24 h urines were collected from three groups of 4, 8

and 4 rats, respectively. Fresh feces were collected from the second group only. The first day of sample collection was designated as "day 1". Samples were collected daily at the same clock time (at 9.00 h) from days 1 up to 6. Samples of day 1 were used to establish basal values. Subsequently, all rats received oral lactulose bolus injections twice daily (at 9.00 and 17.00 h) during three consecutive days (days 2-4). Lactulose solutions were directly injected into the stomach with a plastic syringe and a metallic sonde. Each bolus consisted of 3 ml 22.2 g/100 ml (4 rats), 44.3 g/ 100 ml (8 rats) and 66.5 g/100 ml (4 rats) lactulose solution. The lactulose solutions were freshly prepared every day by diluting a lactulose syrup (50 % (w/w) corresponding with 66.5 g/ 100 ml; Duphar, Weesp, The Netherlands) in tap water. The daily lactulose intakes were 2 x 0.67 g, 2 x 1.33 g and 2 x 2.00 g per rat, respectively.

Influence of oral lactulose administration on polyamines and SCFA in intestinal contents and diamine oxidase activity in intestinal tissue

A group of 4 rats was treated with 2 x 1.33 g lactulose/day according to the same protocol as described above. The rats were killed with diethylether on 15.00 h at day 4; i.e. 6 h after the first lactulose injection on day 4. In addition 3 untreated rats were sacrificed to serve as controls. Small intestine, cecum and colon were removed. Samples of cecum and colon contents and fresh feces were collected. The pH of cecum and colon contents was measured immediately, using neutralit indicator paper (Merck, Darmstadt, Germany). A portion of the contents and feces were stored at -20 °C until analysis for SCFA, polyamines and dry weight percentage. The tissues were extensively rinsed with phosphate buffered saline (PBS; pH 7.4). The small intestine was cut lengthwise, rinsed and divided into 5 equal pieces. Tissues were frozen in liquid nitrogen immediately after rinsing and stored at -20 °C until analysis for diamine oxidase activity.

Analytical methods

Urinary free and acetyl-conjugated polyamines were determined as their heptafluoro-butyryl derivatives using capillary gas chromatography with nitrogen-phosphorus detection.[26] Urinary concentrations were expressed in terms of creatinine, measured with a picric acid method. Total polyamines in feces and in cecal and colonic contents were determined with the same gas chromatographic procedure. Aliquots of 50-100 mg were hydrolyzed in 6 M HCl (120 °C, 18 h). After centrifugation the pellet was washed twice with 1 ml 6 M HCl and further treated as described for urine.[27]

For determination of fecal, cecal and colonic SCFA (acetic, propionic and butyric acids) aliquots of about 200 mg were homogenized. The fatty acids were isolated by vacuum distillation[28] and determined by gas chromatography with flame ionization detection, using a megabore fused silica column.[29]

Diamine oxidase activities of intestinal tissues were determined by a high performance liquid chromatographic method, as described by Biondi et al.[30] The small intestine (5 parts), cecum and colon were homogenized in 10 ml ice cold PBS (pH 7.4) in a Potter-Elvehjem apparatus (Braun, Melsungen, Germany). After centrifugation an aliquot of 0.5 ml (1:30 diluted in PBS) was incubated with 0.03 M cadaverine as substrate (15 min., 37 °C) and

100 µl of 0.1 M acetaldehyde. The reaction was stopped with 10 % trichloroacetic acid and 40 nmol δ-pyrroline was added as internal standard. After reaction with o-aminobenzaldehyde and oxidation with 1 M CrO_3 in 2 M sulphuric acid the resulting quinazolone chromophores were determined by high performance liquid chromatography with spectrophotometric detection at 268 nm.

Protein concentrations in the supernatants of intestinal tissue homogenates were determined by a modified method of Lowry.[31]

Dry weight percentages of cecal and colonic contents and feces were determined by weighing samples before and after freeze drying until constant weight (about 30 h).

Statistical analyses

Statistically significant differences ($P<0.05$) were determined with the paired or unpaired Student's t tests or Mann-Whitney U test each with two-sided probabilities. The employed tests are indicated in the text or legends of the "Results" section.

Results

Influence of oral lactulose administration on urinary polyamine excretion

Daily lactulose dosages of 2 x 0.67 and 2 x 1.33 g/rat, given by gavage, caused soft stools but no diarrhea, whereas a daily dose of 2 x 2.00 g/rat resulted in a mild form of diarrhea.

Rats receiving 2 x 1.33 g lactulose daily excreted increased amounts of particularly spermidine and spermine and to a lesser extent putrescine into their urines (Fig. 1A). In the 24 h of the first two oral lactulose injections (day 2) the increase was 9.9-fold for both spermidine and spermine and 1.7-fold for putrescine, compared with basal levels. After the initial rise the urinary excretion of spermidine and spermine steadily decreased, despite continuous administration of lactulose on days 3 and 4 (Fig. 1A). Cadaverine excretion was not affected. The fecal content of putrescine, cadaverine and spermine was increased from day 3; i.e. the second day of lactulose administration. For putrescine and cadaverine, the highest levels were obtained on day 5, at which the increases amounted to 5.7 and 12.4-fold, respectively (Fig. 1B). Fecal spermidine content decreased during lactulose treatment and recovered after its discontinuation. The fecal SCFA contents decreased as a consequence of lactulose treatment and recovered within 2 days after the last

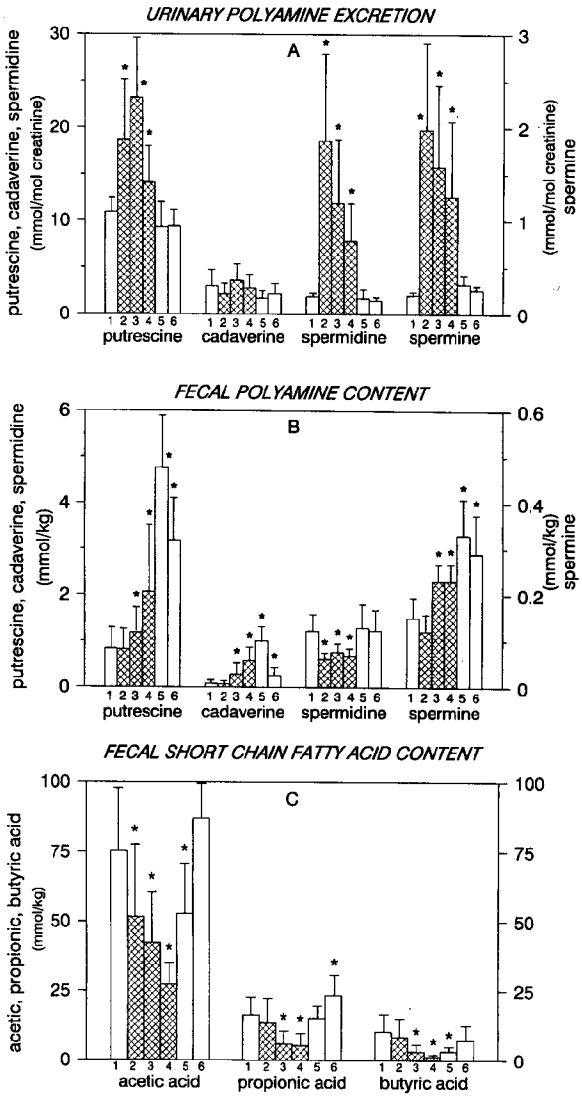

Figure 1. *Influence of orally administered lactulose to rats on urinary polyamine excretion (A), fecal polyamine (B) and short chain fatty acids (C) content.*

Eight rats received lactulose twice daily (2 x 1.33 g/rat; at 9.00 and 17.00 h) by gavage during 3 consecutive days (days 2, 3 and 4; shaded bars). No treatment was given at days 1, 5 and 6 (open bars). Urine and feces were collected throughout. *; indicates statistically significant differences (paired Student's t-test, $P<0.05$) relative to basal values (day 1).

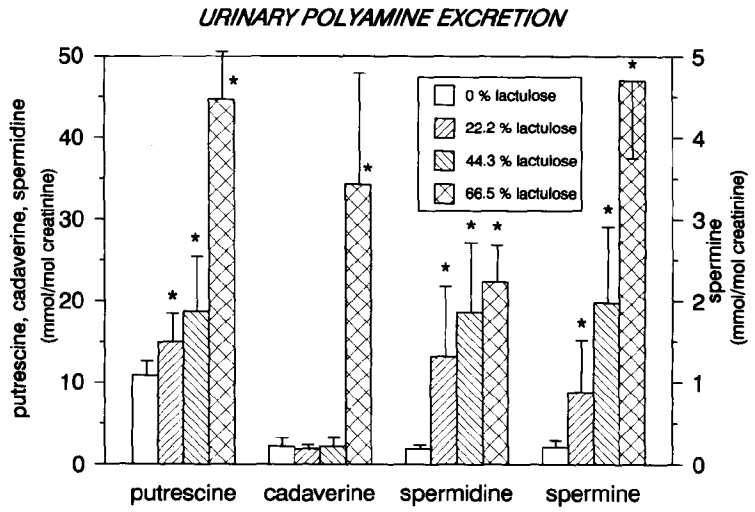

Figure 2. *Dose dependent relation between orally administered lactulose to rats and urinary polyamine excretion.*

Three groups of rats received lactulose given by gavage twice daily at 9.00 and 17.00 h. Data for urinary polyamine excretion are given for the first day after treatment. Another group (n = 8) served as a control. The doses were: 2 x 3 ml 22.2 % lactulose (2 x 0.67 g/rat; n = 4), 2 x 3 ml 44.3 % lactulose (2 x 1.33 g/rat; n = 8), 2 x 3 ml 66.5 % lactulose (2 x 2.00 g/rat; n = 4). Lactulose concentrations and corresponding symbols for urinary polyamine excretion are given in the inset. *; indicates statistically significant differences (unpaired Mann-Whitney U test, $P<0.05$) relative to untreated rats (open bars).

administration (Fig. 1C).

Fig. 2 shows that lactulose treatment caused a dose-dependent increase in the urinary excretion of putrescine, spermidine and spermine, within 24 h of the first two oral injections. Urinary cadaverine excretion increased at a dose of 2 x 2.00 g per day only. For the daily doses of 2 x 0.67 and 2 x 2.00 g lactulose the courses of urinary putrescine, spermidine and spermine excretion were similar to those presented in Fig. 1A (data not shown).

Influence of oral lactulose administration on polyamines and SCFA in intestinal contents and on diamine oxidase activity in intestinal tissue.

The effects of a daily dosage of 2 x 1.33 g lactulose for 2.5 days on pH, dry weight percentage, polyamines and SCFA in the contents of

TABLE I. Influence of oral administration of lactulose on polyamines and SCFA in the contents of cecum and colon and in feces.

	Cecum		Colon		Feces	
Lactulose treatment	-	+	-	+	-	+
Polyamine contents (μmol/kg wet weight)						
Putrescine	55±7	104±21*	52±14	126±46*	830±482	2064±1171*
Cadaverine	11±1	23±11*	10±1	30±19*	82±32	576±269*
Spermidine	156±17	80±16*	159±25	86±6*	1214±327	685±187*
Spermine	19±3	21±5	17±9	27±6	152±41	236±43*
SCFA content (mmol/kg wet weight)						
Acetic acid	60±25	20±7*	57±29	36±21	75±23	27±8*
Propionic acid	12±5	0.4±0.3*	11±6	0.7±0.4*	16±7	5±4*
Butyric acid	17±2	0.5±0.3*	12±5	1.4±1.0*	10±6	1.2±0.6*
Dry weight (%)	22.5±0.9	16.8±1.9*	26.0±1.6	19.5±1.5*	54.0±7.0	29.9±3.7*
pH	7	5.5	7.5	6		

One group of untreated rats (n=3) and a group of lactulose treated rats (n=4) were studied. Lactulose (2 x 1.33 g/day/rat) was administered as an oral bolus injection during 3 days according to the protocol described in "Materials and Methods". Fresh feces and samples of cecum and colon contents were collected. *; indicates statistically significant differences between values from lactulose treated and untreated rats, calculated with the unpaired Mann-Whitney U test ($P<0.05$).

cecum and colon and in feces are shown in table I. In all samples spermidine, SCFA, dry weight percentage and pH decreased, whereas putrescine and cadaverine increased. Spermine increased in feces only. Compared with cecum and colon contents, feces had the highest polyamine levels, irrespective of lactulose treatment. This pattern was not consistently observed for SCFA. Lactulose treatment caused a 25 % decrease in the dry weight percentage of intestinal contents and a 45 % decrease in dry weight percentage of feces. The drop in pH was about 1.5 pH units in both cecum and colon.

A daily dosage of 2 x 1.33 g lactulose for 2.5 days caused no differences in diamine oxidase activities of the small intestine and cecum,

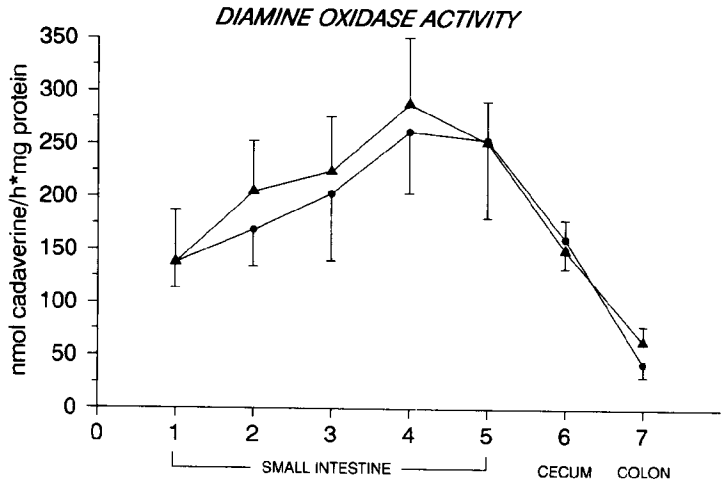

Figure 3. *Tissue diamine oxidase activity along the intestinal tract of the rat.*
One group of untreated rats (●, n = 3) and a group of lactulose treated rats (▲, n = 4) were used for diamine oxidase activity determinations of intestinal tissue. Rats were treated during 3 days with lactulose (see legend figure 1). Small intestines were cut into 5 equal parts from duodenum to cecum. Diamine oxidase activity is expressed as the amount of oxidatively deaminated cadaverine in nmol/h/mg protein.

when compared with untreated rats (Fig. 3). In the colon there was a small increase in diamine oxidase activity (from 43 ± 12 to 64 ± 8 nmol cadaverine/h/mg protein; Mann-Withney U test, $P < 0.05$).

Discussion

The results show a relationship between bacterial diamine (putrescine and cadaverine) production and carbohydrate fermentation activity: oral lactulose treatment caused increased levels of putrescine and cadaverine in feces and in the contents of cecum and colon (Table I). Bacterial lactulose fermentation caused a decrease in cecal and colonic pH from 7 to 5.5 and 7.5 to 6, respectively (Table I), which was also found by others.[14,32] This lowered pH is the optimal pH for induction of microbial biodegradative decarboxylases, e.g. arginine, lysine and ornithine decarboxylases.[2,9,13] It is therefore comprehensible that the amines are

formed by biodegradative decarboxylation to neutralize the produced acids in the bacterial cell, as previously suggested.[13] The decrease in fecal spermidine content (Table I) during the 2.5 days of lactulose administration (from 1.21 ± 0.32 to 0.69 ± 0.19 mmol/kg; i.e. 43 % decrease) coincided with a decrease in fecal dry weight percentage (from 54.0 ± 7.0 to 29.9 ± 3.7 %; i.e. 45 % decrease). A similar relation can be observed for cecum and colon contents (Table I). It suggests that the drop in fecal and luminal spermidine concentration is caused by a decrease in the number of bacteria per gram wet weight. In spite of less bacteria per gram content putrescine and cadaverine levels are even increased. This provides further evidence for the induction of the biodegradative decarboxylases, by which the diamines are formed. Although fecal spermine content was slightly but significantly increased, this was not observed in cecum and colon contents. This increase cannot be ascribed to bacterial changes, since the vast majority of bacteria cannot synthesize spermine. We have no satisfactory explanation for this finding.

The decrease in cecal, colonic and fecal SCFA levels (Table I and Fig. 1C), after daily administration of 2 x 1.33 g lactulose can be ascribed to the resulting decreased pH. A low pH favours the growth of acidophilic bacteria, such as Lactobacilli. These organisms predominantly produce lactic acid from lactulose and thereby discourage growth of bacteria producing SCFA and inhibit the metabolism of organisms which utilize lactic acid.[33]

The most remarkable result is the greatly enhanced urinary polyamine excretion from a daily bolus of as low as 2 x 0.67 g lactulose. Besides putrescine high levels of urinary spermidine and spermine were noted (Fig. 1A). Because of the lack, or very low, activity of spermine synthase in bacteria it does not seem possible that the excess of spermine in urine has been synthesized by bacteria.

It may also be argued that the enhanced urinary excretion of spermidine and spermine is caused by intestinal cell degradation. This is, however, unlikely since such high levels of urinary polyamines have neither been observed in rats during massive liver destruction by tetrachloromethane intoxication[34], nor in patients with high grade non-Hodgkin lymphoma during successful chemotherapy.[35] It is therefore

concluded that the bacterially formed putrescine, is subsequently absorbed and partially converted into spermidine and spermine by eukaryotic cells. It is well established that eucaryotic cells (intestinal cells included) are able to absorb putrescine, as well as spermidine and spermine, via an active uptake mechanism.[24,36,37] The preference to convert putrescine into spermidine and spermine over oxidative deamination at extremely high putrescine influxes from the lumen can be ascribed to the low diamine oxidase activity in the colon, compared with the small intestine (Fig. 3).

Following the sudden increases of urinary spermidine and spermine at day 2 their levels gradually declined at days 3 and 4, in spite of continuous lactulose administration (Fig. 1A). This may be explained by gradually decreasing absorption efficiency by colon cells. Polyamine transport is known to be highly regulated by the polyamine concentration via a negative feed-back mechanism.[37] Furthermore, lactulose acts as a cathartic, which may also decrease the uptake ability. The decreased diamine uptake is supported by increased fecal putrescine and cadaverine excretion from day 3 (Fig. 1B). Another explanation can be found in an enhanced putrescine degradation caused by an apparently moderate induction of diamine oxidase in the colon (Fig. 3), or by increased acetylation. However, in this period (days 2-5) we found no increase in urinary excretion of isoputreanine, a catabolite of spermidine formed by oxidative deamination. Further, we found no alterations in the excretion of N^1-acetylspermidine and N^1-acetylspermine (data not shown).

Induced polyamine production and subsequent excretion in urine during excessive bacterial fermentation may also take place in humans. The beneficial action of oral lactulose administered to patients with portal-systemic encephalopathy has been related to a variety of factors.[38,39] It was recently shown that intraperitoneally injected putrescine is able to reverse the ethanol-associated inhibition of liver regeneration.[40] In another study[41], it was demonstrated that putrescine administered to rats with induced acute liver failure (by D-galactosamine treatment) improves the survival rate of these rats, lowers alanine aminotransferase levels in serum and increases [^{3}H]thymidine incorporation in liver. The latter results together with our data suggest that putrescine formed by the

colonic bacteria during lactulose treatment may be transported to liver cells and may accelerate liver regeneration.

The present results also suggest that polyamines are formed by biodegradative decarboxylation in the colon of neonates. Lactase activity in the small intestine remains low during fetal development until approximately the 36th week of gestation. It subsequently increases continuously until term.[42] Although notably premature infants (with low intestinal lactase activity) exhibit lactose fermentation in their colons[43], full term infants have also been found to ferment lactose to some extent.[44-46] Lactose fermentation in infants is known to lead to the production of lactate[46,47], mainly by lactobacillus bifidus, and a decreased colonic pH.[48] The eventual concomitant production, subsequent absorption and transport of polyamines to the small intestine via an enterohepatic circulation[24] may be beneficial for the development of the intestinal tract[49,50] and possibly other organs.

The process of diamine formation by biodegradative decarboxylases will possibly also take place under normal (non-artificially stimulated) conditions. Especially arginine decarboxylase activity was high in the lumen of colon and cecum of normally fed Sprague-Dawley rats.[24] However, it appears that under normal conditions these polyamines are not excreted in urine, since total GI-tract decontamination of rats did not alter their urinary polyamine excretion (data not shown). Probably, diamine oxidases in bacteria or colon cells prevent the appearance of intestinally formed putrescine and cadaverine in urine. It has been demonstrated that at least part of the oxidatively deaminated products, pyrrolidine and piperidine, respectively, are from intestinal bacterial origin.[21-23] Furthermore, it is possible that the absorbed putrescine is used for the synthesis of structural polyamines in tissues. The uptake of putrescine will become upregulated during polyamine depletion by DFMO treatment.[37] Thus, bacterially formed putrescine in the colon by biodegradative arginine and ornithine decarboxylases is likely to be the major source of exogenous polyamines that abolish the cytostatic effect of DFMO in vivo.[4-7]

Acknowledgements

This study was supported in part by a grant (87-02) from The Koningin Wilhelmina Fonds (Dutch Cancer Society). We thank Hans Peters and Theo de Haan for their valuable technical assistance.

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2.3 The fate of ingested putrescine in rats and mice.

J. Hessels, G. J. de Vries, H. Ferwerda, H. Elzinga,
A. W. Kingma and F. A. J. Muskiet.

Summary

Administration of tetra-deuterated putrescine (Pu-d_4) to rats by gavage (4.3 µmol/rat/day) led to accumulation of Pu-d_4 in a variety of organs in which it is obviously degraded by diamine oxidase. Pu-d_4 was to a negligible extent converted into spermidine (Sd-d_4), but not into spermine (Sp-d_4). A small amount of Pu-d_4 was excreted into urine. Additional inhibition of diamine oxidase by aminoguanidine highly increased urinary excretion of Pu-d_4, but not of Sd-d_4 and Sp-d_4. Inhibition of polyamine oxidase had no effect on Pu-d_4 excretion. Inhibition of endogenous putrescine biosynthesis in GI decontaminated mice resulted in a decrease in intestinal diamine oxidase activity. Under these circumstances intraperitoneally administered Pu-d_4 became efficiently converted into Sd-d_4 (27 % of cellular spermidine) and Sp-d_4 (25 %) in small intestinal cells. Feeding rats diets with different diamine contents caused dose-independent urinary putrescine and reverse dose-dependent cadaverine excretion levels. Additional treatment with aminoguanidine did not show a relationship between urinary putrescine excretion and dietary putrescine content. However, cadaverine excretion levels related to dietary cadaverine content in a positive manner. It is concluded that there are differences in availability and/or metabolic fate between putrescine given by gavage and dietary putrescine. Polyamine uptake from exogenous sources during inhibition of polyamine biosynthesis is discussed.

Introduction

The polyamines spermidine, spermine and the diamine putrescine are present in all mammalian cells.[1] There is general agreement that polyamines are essential for cell proliferation.[2] This notion is especially based on the antiproliferative activity of α-DL-difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase,[3] towards various (tumor) cell lines grown in culture. The cytostatic effect was completely reversible by addition of polyamines to the medium.[4] Under normal circumstances experimental animals have the disposal of large amounts of exogenous polyamines, that are present in the GI tract.[5,6] Recent studies with tumor bearing animals demonstrated that this polyamine pool, originating from the diet and mostly colonic bacteria, is responsible for the abolition of the cytostatic effect of DFMO.[7-9]

We recently reported the uptake, metabolism and urinary excretion of diamines synthesized by colonic bacteria in rats.[5] However, little is as yet known on the fate of polyamines from the diet. Under normal circumstances the high diamine oxidase activity in the small intestinal mucosa is considered to be an efficient barrier, that protects the body against high dietary levels of diamines, e.g. histamine, putrescine and cadaverine.[10,11] A recent study in which labelled polyamines were administered to rats via gastric intubation showed, however, that in the small intestine polyamines are taken up from the lumen.[12] The efficiency of the diamine barrier may be influenced by DFMO treatment, which was shown to prevent the natural increase of mucosal diamine oxidase activity during intestinal maturation of newborn rats.[13]

The objective of this study was to investigate absorption, metabolism (in various organs) and urinary excretion of dietary putrescine. For this, rats were administered 1,1,4,4-tetradeuterated putrescine (Pu-d_4) by gavage and fed diets differing in diamine content. Metabolic pathways were investigated by treatment with aminoguanidine (inhibitor of diamine oxidase) and N,N′-bis(2,3-butadienyl)putrescine (MDL 72527; inhibitor of polyamine oxidase).[14] Intestinal putrescine absorption and metabolism in mice were influenced by inhibition of endogenous *de novo* putrescine

synthesis (treatment with DFMO) and by minimization of the bacterial putrescine pool (GI decontamination).

Materials and methods

Chemicals

Usual laboratory chemicals were from Merck (Darmstadt, Germany). Deuterium labelled putrescine (Pu-d_4; $H_2NCD_2CH_2CH_2CD_2NH_2$) was prepared by catalytic reduction of succinonitrile with deuterium gas. Deuterium labelled spermidine (Sd-d_4; $H_2N(CH_2)_3NHCD_2$-$(CH_2)_2CD_2NH_2$) and spermine (Sp-d_4; $H_2N(CH_2)_3NHCD_2(CH_2)_2CD_2NH(CH_2)_3NH_2$ were synthesized by cyanoethylation of Pu-d4 with acrylonitrile, followed by catalytic reduction of the mono- and dicyanoethyl condensation intermediates with hydrogen gas.[15] DFMO and N,N'-bis(2,3-butadienyl)1,4-diaminobutane dihydrochloride (MDL 72527) were generously donated by the Marion Merrell Dow Research Institute (Strasbourg, France). o-Aminobenzaldehyde (OAB) was prepared by reduction of o-nitrobenzaldehyde.[16] Δ^1-Pyrroline was made by dissolving γ-aminobutyraldehyde diethyl acetal (Janssen, Beerse, Belgium) in 0.01 N HCl.

Animals and diets

Three months old female Wistar rats (150 - 180 g) and DBA-2 mice (18 - 22 g) were used. The mice were obtained from Centrum fur Versuchstiere (Hanover, Germany). All animals were housed under standard laboratory conditions (20-22 °C, 50-60% relative humidity, 12 h-light/12 h-dark cycle) with free access to acidified drinking water (pH 2.5 - 3.0) and RMH-B diet, unless otherwise stated. During the experiments rats were housed individually in metabolic cages. In case of decontamination experiments mice were housed under isolated conditions in sterile cages with sterile sawdust bedding to prevent infection.

Standard rat mouse hamster diet (RMH-B), sterilized rat mouse (SRM-A) diet and a semi-synthetic diet were purchased from Hope Farms (Woerden, The Netherlands). The semi-synthetic diet is a polyamine deficient chow and is further referred to as PDC. The composition of PDC and the contents of supplemented antibiotics for GI decontamination (0.2 g% cefamandol and 0.2 g% kanamycin) and DFMO (3 g%), were essentially the same as previously described.[7,17] The supplemented diets are further referred to as PDC/antibiotics and PDC/antibiotics/DFMO, respectively. Diamine contents of the diets are given in Table III.

Experiments

Urinary polyamine excretion by rats pretreated with PBS, aminoguanidine or MDL 72527, followed by oral Pu-d_4 administration.

Six Wistar rats were divided into 3 groups. Twenty-four hour urine samples were collected during six consecutive days using 50 ml containers each filled with 1 ml 2 M HCl. From day 0 to 5 the control group was daily intraperitoneally (i.p.) injected with 0.5 ml phosphate buffered saline (PBS), containing NaCl (8.0 g/l), KCl (0.2 g/l), Na_2HPO_4 (1.15 g/l) and KH_2PO_4 (0.2 g/l), pH 7.4. The other groups received daily i.p. injections with aminoguanidine (25 mg/kg) or MDL 72527 (20 mg/kg) in PBS. At days 2, 3 and 4 all rats were treated with 6.45 μmol Pu-d_4 (1.5 ml of 4.3 μmol Pu-d_4 /ml distilled water) by gavage, once daily at the same clock time. Naturally occurring and deuterated polyamine concentrations were measured in unhydrolyzed urine.

Tissue polyamine contents of rats after oral administration of Pu-d_4.

A group of 10 Wistar rats was divided into two equal groups. One group remained untreated (control), whereas the other received 1 ml of the Pu-d_4 solution (4.3 μmol/day) by gavage for 4 consecutive days. Rats were killed 4 h after the last Pu-d_4 administration. The whole intestine, liver, spleen and kidney of each rat were removed. The small intestine, cecum and colon were cut lengthwise and washed with PBS. The tissues were immediately frozen in liquid nitrogen and stored at -20 °C until analysis for naturally occurring and deuterated polyamines.

Diamine oxidase activity and Pu-d_4 uptake in the small intestine of polyamine deficient mice.

Twenty-seven mice were fed PDC/antibiotics during 5 days. The mice were divided into 2 groups of which the control group consisted of 10 animals. Subsequently, mice of the treatment group (n = 17) were fed PDC/antibiotics/DFMO during 3 days. Mice of the control and treatment groups were killed with diethyl ether at days 5 and 8, respectively. Their whole intestines were removed and handled as described above. The tissues were used for determination of diamine oxidase activity.

Three groups of DBA2 mice were fed PDC/antibiotics during 5 days. Mice of group A (n = 6) received no further treatment. Mice of group B (n = 5) were fed PDC/antibiotics/-DFMO during another 3 days. The mice were killed with diethyl ether at days 5 and 8, respectively. Mice of group C received the same treatment as those of group B, and were in addition injected i.p. twice daily (at 9 and 17 h) with 1 μmol Pu-d_4 in 0.2 ml PBS during another 2 days. These mice were killed at 16 h after the last injection. Small intestines of all mice were removed and handled as described above. The tissues were used for determination of naturally occurring and deuterated polyamines.

Urinary excretion of polyamines by rats fed SRM-A, RMH-B diets or PDC followed by aminoguanidine treatment.

Three groups of 3,2 and 3 rats were fed SRM-A, RMH-B diet or PDC, respectively, for at least 4 days to accustom the animals to the diets. During 6 consecutive days 24 h urines were collected to establish basal values. Subsequently, all rats received daily i.p. injections

with aminoguanidine (25 mg/kg). From 3 days after the first injection 24 h urines were collected during 4 days to establish treatment values. Unhydrolyzed urines were used for measurement of polyamine concentrations.

Analytical methods

Polyamine concentrations in unhydrolyzed and hydrolyzed urines (6 M HCl, 18 h, 120 °C) were measured as their heptafluorobutyryl derivatives using gas chromatography with nitrogen-phosphorous detection (GC/NP) as previously described.[16,19] For tissue polyamine determination, the samples were homogenized in 10 volumes 6% sulphosalicylic acid and centrifuged. The supernatants were used for polyamine analyses as described for unhydrolyzed urines. Putrescine determined in unhydrolyzed urine and tissue consists of naturally occurring and deuterated putrescine and is further referred to as total putrescine. The same holds for spermidine and spermine.

Deuterium labelled polyamine concentrations were determined by gas chromatography-mass spectrometry (GC/MS), using a Hewlett Packard 5890 gas chromatograph directly coupled to a Trio-II quadrupole mass spectrometer (VG Instruments, Manchester, UK). Operating conditions during selective ion monitoring were essentially the same as previously described.[7] The relative amounts of Pu-d_4, Sd-d_4 and Sp-d_4 were determined by measuring the peak area ratios at m/z 228/226, 540/536 and 580/576 at the retention times corresponding with derivatized putrescine, spermidine and spermine, respectively. Calculation of the ratios between deuterated and naturally occurring polyamines was done by means of a calibration graph, prepared from mass spectrometric data of various combinations of 2 stock solutions containing 70.5 μM putrescine, 30.0 μM spermidine, 5.9 μM spermine and 56.8 μM Pu-d_4, 25.9 μM Sd-d_4, 6.7 μM Sp-d_4, respectively. Using the ratios between labelled and unlabelled polyamines the concentrations of deuterated and naturally occurring polyamines were calculated from the corresponding total polyamine concentrations, as measured with GC/NP.

For the assay of diamine oxidase activity, the entire small intestine was homogenized in 10 ml ice-cold 0.15 M phosphate buffer pH 7.0 with a Potter-Elvehjem apparatus, followed by centrifugation at 2500 g for 15 min. The supernatant was decanted and diluted 30 fold with phosphate buffer. Diamine oxidase activity was assayed as described by Biondi et al.[20], with slight modifications. Briefly, reaction mixtures each consisting of 0.5 ml diluted supernatant, 50 μl 0.03 M cadaverine (substrate) and 100 μl 0.1 M acetaldehyde were prepared. The mixtures were incubated at 37 °C for 15 min. The reaction was stopped with 0.1 ml 10% trichloroacetic acid. After the addition of 40 nmol Δ^1-pyrroline (internal standard) and 50 μl of 0.1 M o-aminobenzaldehyde, the mixtures were heated at 80 °C for 15 min. An aliquot of 0.5 ml of 1 M CrO_3 in 2 M sulphuric acid was added, followed by a second heating at 80 °C for 15 min. The solutions were adjusted to pH 10 and extracted with diethyl ether. After evaporation of the organic phase, the residue was redissolved in 100 μl ethyl acetate. The resulting quinazolone chromophores of Δ^1-piperidine (from cadaverine) and Δ^1-pyrroline were analyzed by straight phase HPLC with UV-detection. Blancs were prepared by incubation of samples in the presence of 1.5 mM aminoguanidine.

Results are expressed in mU/g tissue. One mU is defined as the amount of enzyme that catalyses the oxidation of 1 nmol of cadaverine per min under the described conditions.

Statistical analyses

Statistically significant ($P<0.05$) differences were determined with the Student's t test with a two sided probability.

Results

Urinary polyamine excretion by rats pretreated with PBS, aminoguanidine or MDL 72527, followed by oral Pu-d_4 administration.

As shown in figure 1 (top) oral Pu-d_4 administration to rats led to the appearance of a small amount of Pu-d_4 in urine (0.22 ± 0.16 μmol/24 h; mean ± s.d.; n = 6 urines). Pu-d_4 excretion amounted to about 14 % of total putrescine excretion in 24 h urine, representing less than 4 % of the daily Pu-d_4 dose. The percentage of Sd-d_4 was less than 1 % of total spermidine.

During aminoguanidine treatment baseline urinary putrescine excretion levels increased about 5-fold (from 1.53 ± 0.19 to 8.02 ± 0.47 μmol/24 h; Fig. 1, bottom).

Additional oral administration of Pu-d_4 resulted in a marked increase of total putrescine excretion levels (Fig. 1, bottom), due to the excretion of Pu-d_4 (5.95 ± 1.87 μmol/24 h). The excreted Pu-d_4 amounted to 43 % of total putrescine in 24 h urine, and 92 % of the administered 24 h Pu-d_4 dose. The excretion of Sd-d_4 in this group was 0.096 ± 0.060 μmol/24 h, corresponding with 4 % of total spermidine.

Compared with the control group, treatment with MDL 72527 did not change total urinary putrescine excretion. Additional administration of Pu-d_4 resulted in a urinary Pu-d_4 excretion of 0.29 ± 0.09 μmol/24 h, which was not different from values of the control group. Sd-d_4 was not detectable in urines of these rats.

Urinary Sp-d_4 was below the limit of detection in all groups (less than about 0.3% of total spermine).

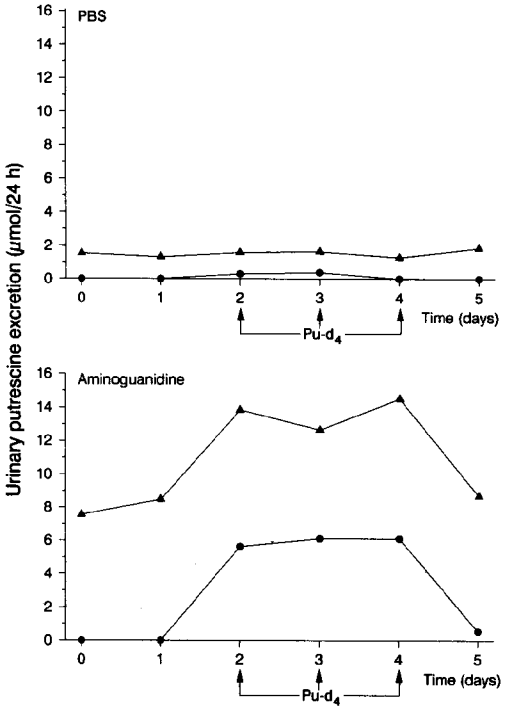

Figure 1. *Urinary putrescine excretion by rats orally treated with tetra-deuterated putrescine (Pu-d_4).*

Urinary excretion of total putrescine (sum of naturally occurring and deuterated putrescine) (▲), and Pu-d_4 (●) by Wistar rats, daily treated by i.p. injections with PBS (top) or aminoguanidine (25 mg/kg; bottom) from day 0 to 5. All rats were given Pu-d_4 (6.45 μmol in 1.5 ml) once daily by gavage at days 2, 3 and 4. Each point represents the mean urinary putrescine excretion for two rats.

Tissue polyamine content of rats after oral administration of Pu-d_4.

Table I shows the influence of oral administration of Pu-d_4 on naturally occurring and deuterated polyamine contents of various rat organs. The treatment resulted in accumulation of about 110 nmol Pu-d_4/g wet weight (range 99-116) in all studied organs. As a consequence of Pu-d_4 accumulation the content of naturally occurring putrescine decreased, whereas total putrescine content increased. Despite the high Pu-d_4 levels Sd-d_4 was present in only marginal amounts (about 0.5% of total spermidine and 3 % of total deuterium labelled polyamines). No Sp-d_4 could be detected. Total spermidine and spermine contents were not

TABLE I: Effect of orally administered Pu-d_4 to rats on tissue polyamine content.

Organ	Pu-d_4 added (oral)	Naturally occurring and deuterated polyamines (nmol/g wet weight)				
		Pu	Pu-d_4	Sd	Sd-d_4	Sp
small intestine	+	85±23	116±9	1306±199	5.1±2.1	502±45
	-	141±22		1501±214		551±28
cecum	+	16±4	109±9	563±41	2.4±0.1	454±8
	-	45±10		601±12		469±21
colon	+	14±4	110±8	632±62	2.8±1.1	451±33
	-	30±3		658±139		456±41
liver	+	1.2±1.1	99±14	708±110	4.4±1.7	992±16
	-	23±6		694±124		947±64
spleen	+	16±2	99±12	1108±71	3.0±0.9	792±21
	-	51±10		1314±213		771±34
kidney	+	5.6±1.4	106±13	465±34	2.2±0.7	723±12
	-	29±3		487±69		756±86

A group of 5 rats received 1 ml of a Pu-d_4 solution by gavage once daily (4.3 µmol/ml) during 4 days. A second group of 5 untreated rats served as controls. Naturally occurring and deuterated putrescine (Pu and Pu-d_4), spermidine (Sd and Sd-d_4) and spermine (Sp) contents of various organs were determined. Sp-d_4 was below the detection limit. Results are presented as means ± S.D.

affected by Pu-d_4 treatment.

Diamine oxidase activity and Pu-d_4 uptake in the small intestine of polyamine deficient mice.

Feeding mice PDC/antibiotics/DFMO, resulted in a marked decrease of intestinal diamine oxidase activity, compared with the DFMO non-treated group. The intestinal diamine oxidase activity in the latter group was 431±40 mU/g wet tissue, whereas the activity found in the DFMO treated group amounted to 227±54 mU/g tissue, corresponding with a decrease of 47 %.

Table II shows that DFMO treatment of decontaminated mice led to a decrease in small intestinal putrescine and spermidine, but not in spermine. Administration of Pu-d_4 (i.p.) during DFMO treatment resulted

TABLE II. Uptake and metabolism of i.p. administered Pu-d4 in small intestinal tissue of polyamine deficient mice

		naturally occurring and deuterated polyamines					
group	treatment	Pu	Pu-d_4	Sd	Sd-d_4	Sp	Sp-d_4
A	dcPDC	89 ± 2		1246 ± 151		847 ± 102	
B	dcPDC/DFMO	21 ± 6		625 ± 64		801 ± 89	
C	dcPDC/DFMO + Pu-d_4	34 ± 13	25 ± 5	673 ± 95	248 ± 42	604 ± 27	204 ± 27

Three groups of DBA2 mice were fed PDC containing antibiotics for GI decontamination (dcPDC) during 5 days. Mice of group A (n = 6) received no further treatment. Mice of group B (n = 5) were fed dcPDC containing DFMO (3 g %) for another 3 days. Mice of group C were in addition twice daily injected i.p. with 1 μmol Pu-d_4 in 0.2 ml PBS for 2 days. Naturally occurring and deuterated polyamines in small intestinal tissues were determined (for abbreviations of polyamines see legend of Table I). Results are presented as means ± S.D.

in appearance of Pu-d_4 (42 % of total putrescine), Sd-d_4 (27 % of total spermidine) and Sp-d_4 (25 % of total spermine) in small intestinal tissue. Compared with DFMO treated mice the total contents of putrescine and spermidine increased, whereas naturally occurring putrescine and spermidine remained constant. The produced Sp-d_4 displaced part of the naturally occurring spermine, resulting in no net changes in total spermine content.

Urinary excretion of polyamines by rats fed SRM-A, RMH-B diet or PDC, followed by aminoguanidine treatment.

Table III shows that feeding rats diets containing increasing polyamine content led to similar putrescine but decreasing cadaverine excretion in urine. Daily i.p. injection with aminoguanidine (25 mg/kg) caused an about 4-fold increase in urinary putrescine, irrespective of the dietary putrescine content. Aminoguanidine treatment did not alter cadaverine excretion levels in the group fed PDC, whereas feeding RMH-B and SRM-A diets resulted in about 3-fold and 7-fold increases of urinary cadaverine excretion, respectively.

TABLE III: Diamine content of diets and their effects on urinary diamine excretion in untreated and aminoguanidine treated rats.

Diet type	Dietary diamines (μmol/g)		Urinary diamine excretion (μmol/24 h)			
			basal		aminoguanidine	
	Pu	Cad	Pu	Cad	Pu	Cad
PDC	0.01	-	2.18 ± 0.54	0.67 ± 0.23	7.66 ± 0.72	0.71 ± 0.16
RMH-B	0.68	0.31	1.85 ± 0.37	0.53 ± 0.18	7.33 ± 0.62	2.23 ± 0.30*
SRM-A	1.47	2.14	1.90 ± 0.65	0.17 ± 0.04*	8.22 ± 1.00*	4.42 ± 0.65*

*Three groups of 3, 2 and 3 rats each received either PDC, RMH-B or SRM-A diet, respectively. After 4 days, 24 h urines were collected during 6 consecutive days to establish basal values. Subsequently, rats were daily treated with aminoguanidine (25 mg/kg; i.p.). After 3 days 24 h urines were collected during 4 days to establish treatment values. Values are means ± S.D. * denotes statistically significant differences ($p \leq 0.05$) relative to PDC fed group.*

Discussion

It is generally assumed that intestinal cells act as efficient polyamine traps owing to their high diamine oxidase activity.[10,23] Catabolism of orally administered Pu-d4 by diamine oxidase seems supported by our results, since high urinary levels of Pu-d_4 were only observed during aminoguanidine treatment (Fig. 1). However, aminoguanidine non-treated rats also exhibited high levels of Pu-d_4 in all studied organs (Table I). The mean total weight of the studied organs was about 17.8 g (liver 7.3 g, spleen 1.8 g, kidneys 1.2 g and gut 7.5 g) and the mean Pu-d_4 content 110 nmol/g. This corresponds with a total Pu-d_4 content of about 2.0 μmol. Thus, relative to the administered Pu-d_4 dose (4.3 μmol/day for 4 days) there is considerable accumulation of Pu-d_4 in tissues. From these results it may be concluded that gastrointestinal diamine oxidase activity is insufficient to completely prevent uptake of orally administered putrescine. Diamine oxidase in other tissues than the small intestine is therefore likely to be responsible for the catabolism of a considerable part of exogenous putrescine. Other catabolic pathways are unlikely. First, because during aminoguanidine treatment almost all (92 %) of the administered Pu-d_4 was recovered from urine as Pu-d_4 and not as e.g. Sd-

d_4 and Sp-d_4. Second, because during polyamine oxidase inhibition the administered Pu-d_4 was not converted into N^1-acetylspermidine, the major excretory form of spermidine under these conditions[22], since no Sd-d_4 was identified in hydrolyzed urines of these rats (data not shown).

A second mechanism possibly involved in the small intestinal polyamine trap relates to the high proliferation rate of the small intestinal lining. Since proliferating cells need polyamines it may be assumed that exogenous putrescine is taken up for the synthesis of higher polyamines. It is, however, remarkable that after 4 days of treatment the accumulated Pu-d_4 in the small intestine is to a negligible extent converted into Sd-d_4 and Sp-d_4 (Table I). This was previously found when rats were given [^{14}C]-labelled polyamines by gavage during a 1 h labelling period.[12] It may be assumed that considerable cell renewal in the small intestine, and thus need for polyamines, occurred during the 4 days treatment period. Obviously, putrescine from exogenous sources is preferably degraded instead of being used for biosynthesis of spermidine and spermine. Separation between these processes by cellular compartmentation, as previously suggested[1,24], is likely to account for this result. The observation that following DFMO treatment a considerable amount of i.p. injected Pu-d_4 is converted into Sd-d_4 and Sp-d_4 in the small intestine (Table II), suggests that, during imminent polyamine deficiency, putrescine may be transported from the intracellular catabolic compartment into the biosynthetic compartment. Furthermore, it is known that DFMO treatment causes decreased diamine oxidase activity and increased intracellular concentrations of decarboxylated S-adenosylmethionine. This may contribute to the conversion of putrescine to spermidine and spermine, instead of its degradation by diamine oxidase. We have no explanation for the addition of the synthesized Sd-d_4 to the existing spermidine pool, as opposed to the partial displacement of spermine by Sp-d_4.

It may be argued that the observed differences in Pu-d_4 metabolism (i.e. degradation under normal conditions and conversion into higher polyamines during DFMO treatment) is caused by different routes of administration, since Pu-d_4 was given by gavage to DFMO non-treated rats and by i.p. injection to DFMO treated mice. This is, however,

unlikely, because we have previously demonstrated that orally administered Pu-d_4 is also converted into Sd-d_4 and Sp-d_4 in L1210 cells of DFMO treated mice.[7]

The finding that during diamine oxidase inhibited conditions orally administered Pu-d_4 is for the greater part excreted in urine (Fig. 1) is inconsistent with the comparable putrescine excretion levels of aminoguanidine-treated rats receiving diets with highly different putrescine content (Table III). The absence of a dose-response relation between dietary putrescine content and urinary putrescine excretion cannot be accounted for by increasing excretion of spermidine, spermine or their acetylconjugates, since their urinary excretion was constant (data not shown). Mentioned discrepancy between the fate of dietary putrescine and putrescine given by gavage can also not be ascribed to differences in the daily intakes. The putrescine intake from the SRM-A diet (about 22 μmol/day, assuming a daily food intake of 15 g/rat), was about 3.4 times higher than the Pu-d_4 dose given by gavage (6.45 μmol/day). Further, it can not be caused by delivery of putrescine in an empty stomach, because Pu-d_4 administration via drinking water yielded similar results (data not shown).

Thus, the present results do not show the destination of dietary putrescine. From the comparison with orally administered putrescine it seems that under normal conditions dietary putrescine is either much less available for absorption, or completely degraded by intestinal diamine oxidase. The latter would be in line with the reverse relation between dietary and urinary cadaverine, and their positive relationship under diamine oxidase inhibited conditions (Table III). It can, however not be excluded that part of dietary putrescine becomes absorbed and converted into higher polyamines, at least during diamine oxidase inhibition. Whatever the fate of dietary putrescine, it is clear that results from orally administered putrescine cannot be used for the study of dietary putrescine.

It was demonstrated that dietary polyamines are partly able to abolish the cytostatic effect of DFMO[7-9], although to much lesser extent than polyamines produced by colonic bacteria.[7] It is therefore conceivable that, in analogy with orally administered Pu-d_4, at least part of dietary

putrescine is absorbed during cellular polyamine deficiency and used for biosynthesis of spermidine and spermine. Apart from possible upregulation of polyamine transport[25,26], the present results suggest that this may additionally be accomplished by decrease in small intestinal diamine oxidase activity.

In conclusion, it appears that the fate of dietary putrescine is different from that of cadaverine and from orally administered putrescine in solution. Since we have no satisfactory explanation for these differences, further studies are necessary to elucidate the fate of dietary putrescine.

Acknowledgement

This study was supported in part by a grant (nr. 87-02) from The Koningin Wilhemina Fonds (Dutch Cancer Society).

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